

# Development of *Candida*-Specific Real-Time PCR Assays for the Detection and Identification of Eight Medically Important *Candida* Species

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**ABSTRACT:** Culture-based identification methods have been the gold standard for the diagnosis of fungal infection. Currently, molecular technologies such as real-time PCR assays with short turnaround time can provide desirable alternatives for the rapid detection of *Candida* microbes. However, most of the published PCR primer sets are not *Candida* specific and likely to amplify DNA from common environmental contaminants, such as *Aspergillus* microbes. In this study, we designed pan-*Candida* primer sets based on the ribosomal DNA-coding regions conserved within *Candida* but distinct from those of *Aspergillus* and *Penicillium*. We demonstrate that the final two selected pan-*Candida* primer sets would not amplify *Aspergillus* DNA and could be used to differentiate eight medically important *Candida* pathogens in real-time PCR assays based on their melting profiles, with a sensitivity of detection as low as 10 fg of *Candida* genomic DNA. Moreover, we further evaluated and selected species-specific primer sets covering *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, and *Candida dubliniensis* and show that they had high sensitivity and specificity. These real-time PCR primer sets could potentially be assembled into a single PCR array for the rapid detection of *Candida* species in various clinical settings, such as corneal transplantation.

**KEYWORDS:** specific PCR, primer design, species differentiation, ribosomal DNA, melting profiles

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## Introduction

Fungal microbes such as those of *Candida* species are considered as part of normal human microbial flora.<sup>1</sup> However, immunocompromised individuals such as recipients of organ or tissue transplants are particularly prone to fungal infections due to the suppression of their immune system. Especially, severe adverse events such as endophthalmitis with *Candida* infections following penetrating keratoplasty in the corneal transplant recipients appear to be increasingly reported.<sup>2,3</sup> The most commonly reported *Candida* species from clinical human cases are *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida guilliermondii*, *Candida lusitanae*, *Candida dubliniensis*, and *Candida krusei*.<sup>4,5</sup>

Most of the well-established laboratory tests for the diagnosis of *Candida* infections are culture-based methods, which are time consuming and laborious. Recent studies have shown that the advances in molecular technologies, such as real-time quantitative PCR (qPCR) assays, are useful in detecting microbial infections without the need for microbial isolation from

the examined samples. Several qPCR assays have been previously developed to detect *Candida* DNA in various clinical settings.<sup>6</sup> Among the target sequences of choice, nuclear ribosomal DNA (rDNA) has been the most commonly used since it exists in the genome as tandemly repeated copies, and the first and second internal transcribed spacers (ITS-1 and ITS-2) of rDNA have been shown to provide reliable species-specific genetic markers for fungal pathogens.<sup>7,8</sup> The rationale of this approach is that the primer sets targeting the sequences of highly conserved rDNA-coding regions (18S, 28S, or 5.8S) that flank ITS-1 or ITS-2 can effectively amplify rDNA from all *Candida* species. Subsequently, the amplicons containing species-specific sequences of the ITS-1 or ITS-2 could be distinguished from each other by restriction fragment length polymorphism,<sup>9</sup> by the use of species-specific Taqman probes or by melting curve analysis using SYBR Green dye at the end of the final real-time PCR cycle for species differentiation.<sup>10</sup> It is of important clinical relevance to distinguish *Candida* microbes to the species level given their different susceptibilities to different antifungal drugs.<sup>11,12</sup>



As most of the previously published pan-fungal primer sets are predicted to amplify a wide range of fungal DNAs based on sequence alignments, in this study, we specifically designed the in-house pan-*Candida* primer sets such that they were predicted not to amplify DNA from *Aspergillus* microbes, common environmental contaminants that can potentially produce undesirable false-positive signals in *Candida*-specific PCR assays. We evaluated the specificity and sensitivity of these primer sets using conventional and real-time PCR assays for the detection and differentiation of eight medically important *Candida* pathogens, namely *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae*, *C. krusei*, *C. guilliermondii*, and *C. dubliniensis*.

## Materials and Methods

**Microbial strains and cultivation.** All fungal species used in this study were obtained from the American Type Culture Collection (ATCC). A total of 16 *Candida* strains representing eight *Candida* species were obtained: *C. albicans* (ATCC 14053, ATCC 11006, and ATCC 18804), *C. glabrata* (ATCC 2001 and ATCC 66032), *C. tropicalis* (ATCC 750 and ATCC 66029), *C. dubliniensis* (ATCC MYA-646 and ATCC MYA-578), *C. krusei* (ATCC 14243 and ATCC 32196), *C. parapsilosis* (ATCC 22019 and ATCC 90018), *C. guilliermondii* (ATCC 6260), and *C. lusitaniae* (ATCC 34449 and ATCC 42720). In addition to the *Candida* species, two *Aspergillus* species, *Aspergillus fumigatus* (ATCC 1066) and *Aspergillus flavus* (ATCC 9643), were included for cross-reactivity testing. All fungal strains were cultured on Sabouraud's dextrose (SD) agar or in SD broth at 30°C for 48 hours for *Candida* species and 72 hours for *Aspergillus* species. The bacterial species, *Streptococcus pyogenes* (ATCC 19615), *Streptococcus pneumoniae* (ATCC 49619), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Clostridium perfringens* (ATCC 3628 and ATCC 13124), *Bacillus cereus* (ATCC 31430), and *Escherichia coli* (ATCC 25922), were obtained from ATCC, cultured following the protocols recommended by ATCC, and used for cross-reactivity testing as well.

**Isolation of genomic DNA.** To isolate fungal genomic DNA, five loops of fungal biomass from SD agar plates were scraped, suspended, and washed extensively in phosphate-buffered saline. The cell pellet was then suspended in YD Digestion Buffer with 25 U of R-Zymolyase (Zymo Research) and incubated at 37°C for one hour. After vortexing thoroughly, the genomic DNA was extracted using ZR Fungal/Bacterial DNA Kit (Zymo Research) according to the manufacturer's protocol. Bacterial genomic DNAs were isolated using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. Human genomic DNA was prepared from the buffy coat of a healthy blood donor from the National Institutes of Health Clinical Center Blood Bank using the DNeasy Blood & Tissue Kit (Qiagen). All DNA samples were quantified using a Nanodrop

Spectrophotometer (Thermo Scientific) and stored at -20°C prior to PCR amplification.

**Pan-*Candida* primer design.** The sequences of 18S, 5.8S, and 28S rDNA of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. krusei*, *C. lusitaniae*, *C. guilliermondii*, *A. flavus*, *A. fumigatus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus penicillioides*, *Aspergillus terreus*, and *Penicillium chrysogenum* were downloaded from the National Center for Biotechnology Information and aligned. Regions of DNA sequences that are conserved within *Candida* species but distinct from *Aspergillus* and *Penicillium* species were identified. A total of four pan-*Candida* primer sets were designed: two primer sets (18S-1F/5.8S-1R and 18S-2F/5.8S-1R) for amplifying ITS-1, one primer set (5.8S-1F/28S-1R) for amplifying ITS-2, and one primer set (28S-2F/28S-2R) for amplifying a variable segment of 28S ribosomal gene were designed. Nucleotide sequences of four in-house designed primer sets, and the published primer sets used in this study are listed in Table 1. A schematic representation of the relative positions of 18S-1F, 5.8S-1R, 5.8S-1F, and 28S-1R primers and two previously published primer sets (Fungal-7a,7b/RT1 and UNF1/UNF2)<sup>13,14</sup> in the rDNA transcriptional unit is shown in Figure 1.

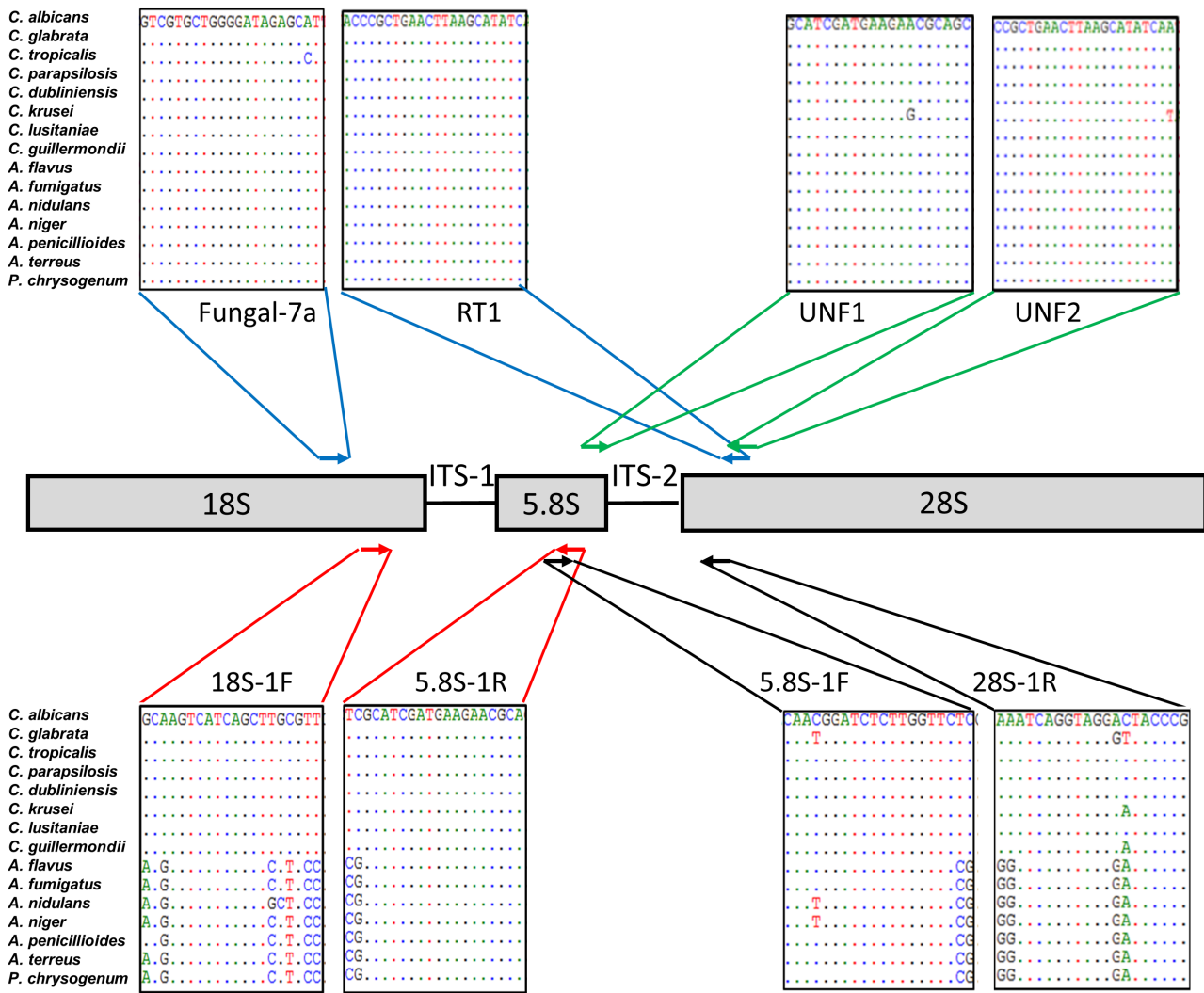
**Conventional PCR testing.** Each PCR mixture contained 10× PCR buffer (100 mM Tris-HCl [pH 9.0], 15 mM MgCl<sub>2</sub>, 500 mM KCl, and 1.0% Triton X-100), dNTPs (2.5 μM), primers (10 μM each), Hotstar Taq polymerase, and DNA template. Amplification was carried out in Mastercycler Pro S (Eppendorf) at 95°C for five minutes, then 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds for 45 cycles, followed by final extension at 72°C for seven minutes. PCR products were analyzed by electrophoresis. Amplicons were detected on ultraviolet transillumination by an electronic documentation system (GelDoc-It Imaging System; UVP).

**Real-time PCR testing and melting curve analysis.** To perform real-time qPCR assays, Power SYBR Green PCR Master Mix containing AmpliTaq gold DNA polymerase (Bio-Rad) was used. Real-time PCR was performed in triplicates using the CFX96 Real-Time PCR Detection System (Bio-Rad) with the following parameters: an initial activation of AmpliTaq gold DNA polymerase at 95°C for 10 minutes, followed by 50 cycles of 95°C for 30 seconds (denaturation) and 60°C for one minute (annealing and extension). For melting curve analysis, amplicons were subjected to melting curve analysis by gradually increasing the temperature from 65 to 95°C at 0.1°C/s and recording the changes in fluorescence intensities to determine the melting profiles for each target amplicon. The threshold of each PCR is determined by the CFX Manager Software (Bio-Rad) with default setting. For each PCR run, a panel of DNA samples consisted of 5 pg, 1 pg, 500 fg, 100 fg, 50 fg, 10 fg, and 5 fg of DNA from each of the target *Candida* species and 5 ng of DNA from *Aspergillus*, common bacteria, and humans, and a no template

**Table 1.** Evaluation of pan-*Candida* primer sets by 2 PCR platforms.

PRIMER <sup>a</sup>	FORWARD PRIMER	REVERSE PRIMER	CONVENTIONAL PCR		REAL-TIME PCR		PRIMER REFERENCE
			SENSITIVITY <sup>b</sup> (fg/REACTION)	SPECIFICITY <sup>c</sup>	SPECIES DIFFERENTIATION	SENSITIVITY <sup>b</sup> (fg/REACTION)	
<b>18S-1F/ 5.8S-1R</b>	GCAAGTCATCAGCTTGCCTT	TGGGTTCTTCATCGAIGCGA	50 (100 for <i>C. glabrata</i> )	0	Yes	10 (100 for <i>C. glabrata</i> )	0 This study
18S-2F/ 5.8S-1R	ACTACCGATTGAATGGCTTA	TGGGTTCTTCATCGAIGCGA	50 (100 for <i>C. glabrata</i> )	0	No	10 (100 for <i>C. glabrata</i> )	0 This study
<b>5.8S-1F/ 28S-1R</b>	CAACGGATCTCTTGGTTCTC	CGGGTAGTCTTACCTGATTT	50 (100 for <i>C. glabrata</i> )	0	Yes	10 (100 for <i>C. glabrata</i> )	0 This study
28S-2F/ 28S-2R	CGGCGAGTGAAGCGGCTAA	ATTCCCAACAACACTCGACTC	50 (100 for <i>C. glabrata</i> )	0	No	10 (100 for <i>C. glabrata</i> )	0 This study
ITS1/ ITS4	TCCGTAGGTGAACCTGCGG	TCCTCCGCTTATTGATATGC	1000	1	n.d.	n.d.	n.d. Dunyach C et al 2008
Cand-F/ ITS-R	CCTGTTTGAGCGTCRITTT	TCCTCCGCTTATTGATAT	100 (1000 for <i>C. albicans</i> )	0	n.d.	n.d.	n.d. Schabereiter-Gurtner et al 2007
Fungal- 7a,7b/RTI	7a:GTCGTGCTGGGGATARAGCAT 7b:GTCGTGCTGGGGATAGARCAT	GATATGCTTAAGTTCAGCGGGT	100	1	No	50	n.d. Mandviwala et al 2010
CandUn/ FungUn	CATGCCCTGTTGAGCGTC	TCCTCCGCTTATTGATATGCT	n.d. <sup>d</sup>	1	No	10	n.d. Goldschmidt et al 2012

**Notes:** <sup>a</sup>Primer names in boldface are the selected primer sets in this study. <sup>b</sup>PCR detection sensitivity for all 8 targeted *Candida* species except for the species in the parenthesis. <sup>c</sup>Specificity: Test was performed using the primer set to amplify 5 ng of genomic DNA prepared from each sample of a control panel containing 2 *Aspergillus* sp., 7 common bacteria and human PBMC. Number "0" indicates no PCR product was amplified from any of the samples from the control panel. Number "1" indicates no PCR product was amplified from any of the samples from the control panel, except the DNA samples from the 2 *Aspergillus* sp. <sup>d</sup>n.d. = not done.



**Figure 1.** Schematic representation of rDNA transcriptional unit, including the 18S, 5.8S, and 28S genes and the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively), showing the relative positions of the primer sets used in PCR. Bases identical to those of *C. albicans* are represented by a dot (.). The primer sequences are listed in Table 1.

control was included. Multiple strains within the same species were used when available.

## Results

**Evaluation of pan-*Candida* primer sets by conventional PCR assays.** The PCR sensitivity and specificity of four in-house pan-*Candida* primer sets were first evaluated using conventional PCR assays (Table 1). The results showed that four in-house designed primer sets detected 50 fg of genomic DNA for all tested *Candida* species, except *C. glabrata*; the limit of detection for the latter is 100 fg of genomic DNA (Table 1). No nonspecific amplicons were observed from any control samples tested upon examination on agarose gel electrophoresis. For comparison, we also tested three previously published pan-fungal primer sets. Primer set ITS-1/ITS-4<sup>7</sup> could detect only 1 pg of genomic DNA from all *Candida* species tested and produced a nonspecific amplification from *Aspergillus* DNA. Primer set Fungal-7a,7b/RT1 detected

100 fg of genomic DNA of all *Candida* species tested and produced a nonspecific amplification from *Aspergillus* DNA. Primer set Cand-F/ITS-R<sup>15</sup> detected 100 fg of genomic DNA of all *Candida* species tested, except *C. albicans*, for which the sensitivity of detection is 1 pg of genomic DNA (Table 1).

**Evaluation of pan-*Candida* primer sets by real-time PCR assays.** We first evaluated the sensitivity of the pan-*Candida* primer sets. The results indicated that all four in-house pan-*Candida* primer sets could detect 10 fg of genomic DNA of all *Candida* species tested, except *C. glabrata*, for which the sensitivity is 100 fg of genomic DNA (Table 1). In addition, the detection sensitivity of the four in-house pan-*Candida* primer sets was not affected by the presence of 5 ng of human DNA in the background (data not shown). For comparison, we also tested the sensitivity of published primer set Fungal-7a,7b/RT1 and CandUn/FungUn,<sup>16</sup> which could detect 50 and 10 fg of genomic DNA, respectively (Table 1). The specificity of four in-house pan-*Candida* primer sets was



tested against a panel of 5 ng of genomic DNA from each of the control samples, including *Aspergillus*, humans, and common bacteria. None of the four in-house primer sets amplified DNA from any control sample (Table 1).

We then evaluated the abilities of the pan-*Candida* primer sets to distinguish the eight medically important *Candida* species by their specific amplicon melting profiles. For pan-*Candida* primer set 5.8S-1F/28S-1R targeting ITS-2, most of the species displayed a single major peak, whereas *C. parapsilosis* appears to have several domains when melted, with a major peak at 79.5°C (Fig. 2). The melting peaks for *C. glabrata*, *C. dubliniensis*, and *C. guilliermondii* seem to be clearly separated from one another; however, there are slight variations from different strains within each species that may be too close to make reliable distinction for species differentiation among themselves. For *C. tropicalis*, *C. albicans*, *C. lusitaniae*, and *C. krusei*, their unique melting profile allows species-specific differentiation with the main peak at 79.0, 81.3, 81.9 and 85.1°C, respectively.

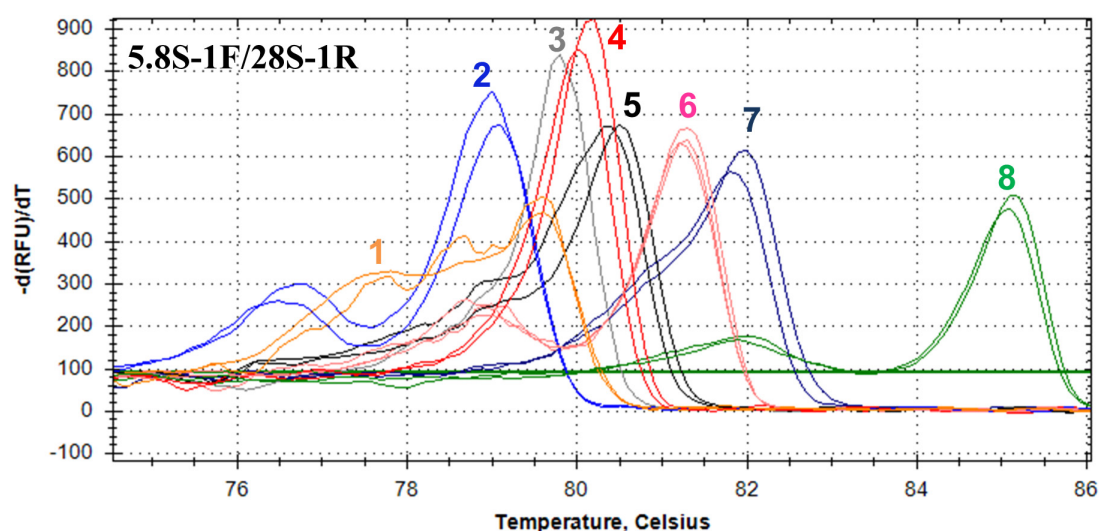
For pan-*Candida* primer set 18S-1F/5.8S-1R targeting ITS-1, the melting profiles for species with two and three melting peaks are shown separately in Figure 3A and B, respectively. For species with two melting peaks, *C. parapsilosis*, *C. tropicalis*, and *C. guilliermondii* could be easily distinguishable from each other by its own unique minor and major peaks. Both *C. dubliniensis* and *C. albicans* had major melting peaks at 81.4–81.5°C, but the separation of the left shoulder of the melting curve may allow distinction between the two species (Fig. 3A). For species with three melting peaks, *C. glabrata*, *C. lusitaniae*, and *C. krusei* could be clearly distinguished from one another by their unique melting profiles (Fig. 3B).

The results using the other two in-house pan-*Candida* primer sets (18S-2F/5.8S-1R and 28S-2F/28S-2R) and the

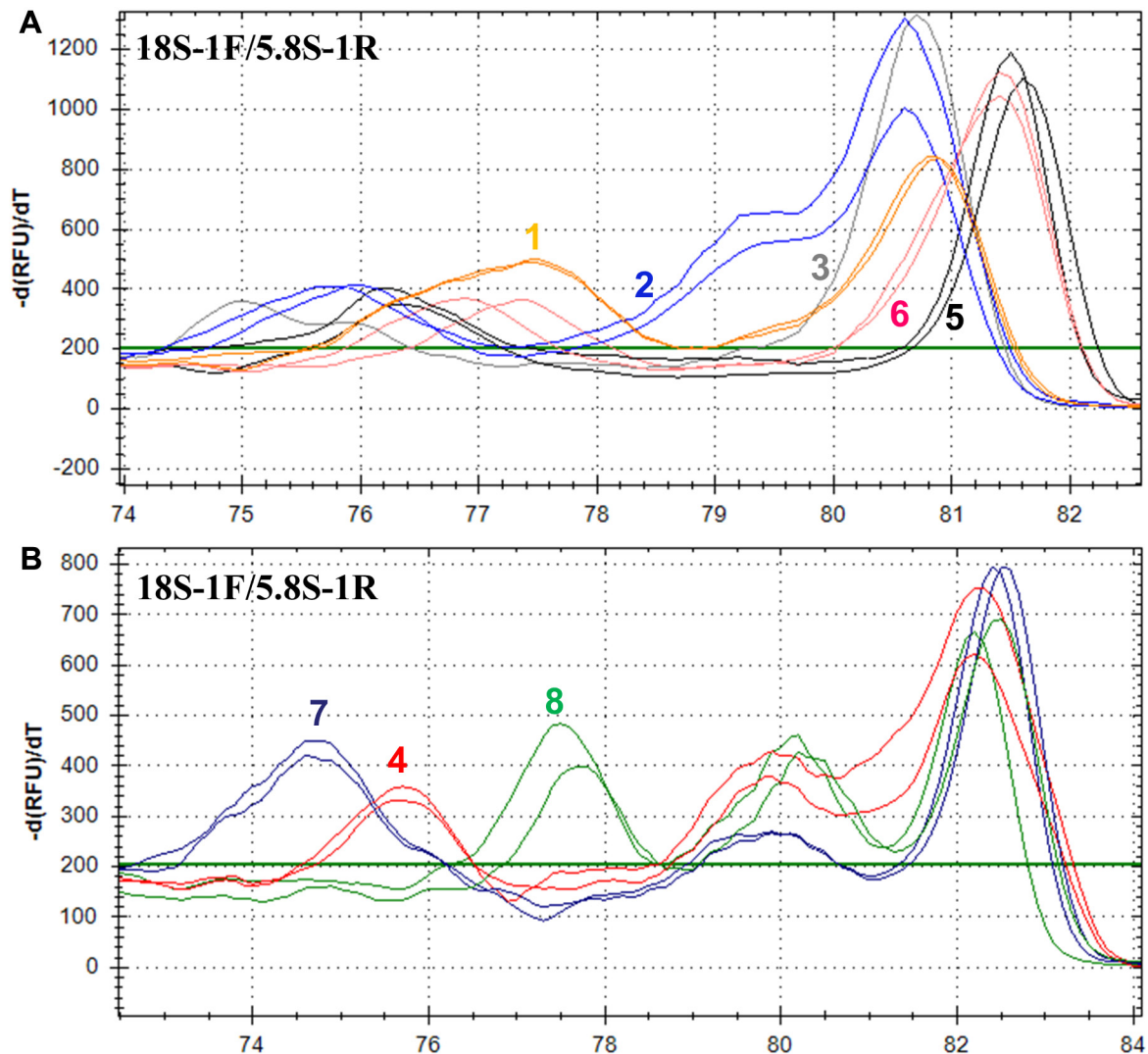
published primer sets CandUn/FungUn and Fungal-7a,b/RT1 are not shown, given the lack of clear distinction in melting profiles for several *Candida* species. The other two primer sets ITS-1/ITS-4 and Cand-F/ITS-R had lower sensitivity of detecting 100 fg to 1 pg of genomic DNA in conventional PCR assay and were not tested in real-time PCR assays.

**Evaluation of species-specific primer sets.** A total of 25 species-specific primer sets (Table 2) were selected either based on the species-specific regions identified from the rDNA sequence alignment or from previously published articles.<sup>8,17,18</sup> We first evaluated the sensitivity and specificity of these primer sets in conventional PCR assays. After excluding the primer sets that had low sensitivity and specificity, we selected 10 primer sets for further testing in real-time PCR assays. As shown in Table 2, the sensitivity for most of the primer sets ( $n = 7$ ) is 50 fg of genomic DNA. Primer set CDU1/2 for detecting *C. dubliniensis* had sensitivity at 100 fg of genomic DNA, while primer set albi 2F/2R for detecting *C. albicans* and primer set trop 3F/3R for detecting *C. tropicalis* had a sensitivity at 500 fg of genomic DNA. In most cases, the detecting sensitivity was not affected by the presence of 5 ng of human DNA in the background (data not shown). Generally, the real-time PCR assays did not produce nonspecific side amplification against other control DNA samples. However, because primer set CTR 5/6 for detecting *C. tropicalis* also amplified genomic DNA of *C. guilliermondii* and *C. krusei* with similar peak, it was, therefore, considered as a nonspecific primer set. Similarly, primer set CPA1/2 for detecting *C. parapsilosis* was also considered as a nonspecific primer set since it also amplified *Aspergillus* genomic DNA with similar peak.

Finally, a total of eight species-specific primer sets covering *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. dubliniensis* with



**Figure 2.** Melting profiles for amplicons targeting ITS-2 using primer set 5.8S-1F/28S-1R. Each profile tracing represents the result from an individual strain of the following tested species: 1. *C. parapsilosis* (2), 2. *C. tropicalis* (2), 3. *C. guilliermondii* (1), 4. *C. glabrata* (2), 5. *C. dubliniensis* (2), 6. *C. albicans* (3), 7. *C. lusitaniae* (2), and 8. *C. krusei* (2). The number in the parenthesis after the species name indicates the number of strains examined in the study.



**Figure 3.** Melting profiles for amplicons targeting ITS-1 using primer set 18S-1F/5.8S-1R. Each profile tracing represents the result from an individual strain of the following tested species: in panel (A), 1. *C. parapsilosis* (2), 2. *C. tropicalis* (2), 3. *C. guilliermondii* (1), 5. *C. dubliniensis* (2), and 6. *C. albicans* (2); in panel (B), 4. *C. glabrata* (2), 7. *C. lusitanae* (2), and 8. *C. krusei* (2). The number in the parenthesis after the species name indicates the number of strains examined in the study.

high sensitivity and specificity were selected (Table 2). These species-specific primer sets are intended to be incorporated with pan-*Candida* primer sets and will be arranged onto a PCR array for the detection and identification of *Candida* species.

### Discussion

Rapid and accurate identification of *Candida* species in clinical specimens is essential for patient care for the initiation of proper antifungal treatments. The traditional culturing methods for the identification of fungal species are time-consuming and the results can sometimes be inconclusive. Therefore, it is highly desirable to develop a robust molecular method for the detection and identification of *Candida* species. Studies using high-resolution DNA melting analysis to discriminate various *Candida* species based on the sequence variation of the target amplicons have been reported previously.<sup>13,17,19</sup>

However, many of these primer sets are also known to amplify *Aspergillus* species, which are common environmental contaminants. Therefore, we specifically designed the pan-*Candida* primer sets in the rDNA regions where the primer sequences are conserved within *Candida* species but distinct from *Aspergillus* species at the 3' end. Indeed, none of our four in-house pan-*Candida* primer sets amplified a large excess of *Aspergillus* DNA (1–100 ng) (cf., the sensitivity of pan-*Candida* primer sets at 10–100 fg of *Candida* DNA), thereby significantly reducing the likelihood of false positivity due to the presence of *Aspergillus* microbes.

The species-level differentiation among *Candida* microbes relies on the distinct melting profiles of the PCR amplicons, which reflects sequence differences within the target regions among different species. In general, the ideal size for amplicons should be less than 150 bp to ensure that the maximum PCR efficiency is achieved.<sup>20</sup> However, it was

**Table 2.** Evaluation of species-specific primer sets by 2 PCR platforms.

PRIMER <sup>a</sup>	TARGET SPECIES	FORWARD PRIMER	REVERSE PRIMER	CONVENTIONAL PCR		REAL-TIME PCR		PRIMER REFERENCE
				SENSITIVITY (fg/REACTION)	SPECIFICITY <sup>b</sup> (fg/REACTION)	SENSITIVITY (fg/REACTION)	SPECIFICITY <sup>b</sup>	
<b>CALB 1/2</b>	<i>C. albicans</i>	TTTATCAACTTGTACACACCAGA	ATCCGGCCTTACCACACTACCG	50	0	50	0	Hsu et al 2003
CALB 3/4	<i>C. albicans</i>	CCTGCCGCCAGAGTCT	CGGTAAACGTCACACACGTATA	Primer dimer	–	n.d. <sup>c</sup>	n.d.	In-house
CALB 5/6	<i>C. albicans</i>	ACTGCTCAAACCATCTCTGG	CACAAGGCCAAATGAAAGGAAT	No amplification	–	n.d.	n.d.	Sardi et al 2011
albi 1F/1R	<i>C. albicans</i>	AGGGAAGCTTGCAGTATTGACAA	ACCAATGCACCTTACTTCATCTC	Primer dimer	–	n.d.	n.d.	In-house
<b>albi 2F/2R</b>	<i>C. albicans</i>	TGGCATCGGCTCTATCGATTATCT	TGACAAGATTAGCGTAAATTCGAG	500	0	500	0	In-house
albi 3F/3R	<i>C. albicans</i>	GACTCAATTTAATCGTCTGGTGGCT	AGTTCCTTAATTAATCGCCGCATGT	No amplification	–	n.d.	n.d.	In-house
<b>CGL 1/2</b>	<i>C. glabrata</i>	TTATCACACGACTCGACACT	CCCACATACTGATGGCCCTACAA	50	0	50	0	Hsu et al 2003
gla 1F/1R	<i>C. glabrata</i>	TTTCTCCTGCCTGCGCTTAAG	GTGTGCGTGGATCTCTCTATTCCA	50	1	n.d.	n.d.	In-house
gla 2F/2R	<i>C. glabrata</i>	ACAGGGTCTGATCAGCAACCA	GCCAACTATCCCAACTGGTGT	No amplification	–	n.d.	n.d.	In-house
<b>gla 6F/6R</b>	<i>C. glabrata</i>	TCGAACTCCTGTTACGAACTTTT	TATGTGGTAGTACCTGAGTTGCAC	50	2	50	0	In-house
<b>gla 8F/8R</b>	<i>C. glabrata</i>	AGGCCCGTTAAAGTAAAGGTCG	GGGGTAGAAGCGTTACTCGTT	50	2	50	0	In-house
CTR 1/2	<i>C. tropicalis</i>	CAATCCTACCGCCAGAGGTTAT	TGGCCACTAGCAAAAATAAGCGT	50	2	n.d.	n.d.	Hsu et al 2003
CTR 3/4	<i>C. tropicalis</i>	CACCCAAACAATTACCAAGT	TGCAAACTCTTTACCTGGAT	No amplification	–	n.d.	n.d.	Sardi et al 2011
CTR 5/6	<i>C. tropicalis</i>	ATTTAACGTGGAACCTATTTTAAGCG	ATCCAGGCCTATGTAAGTTCCCTT	50	2	50	2	In-house
<b>CTR I/anti5</b>	<i>C. tropicalis</i>	CAATCCTACCGCCAGAGGTTAT	CGCTTAAAATAAGTTCCACGTTAAAT	50	0	50	0	CTR1: Hsu et al 2003; anti5: in-house
trop 1F/1R	<i>C. tropicalis</i>	AATGTATCACCGGCAGCCCAAT	CTTGTAATTTCAATGCTGGGGAACA	No amplification	–	n.d.	n.d.	In-house
trop 2F/2R	<i>C. tropicalis</i>	CAGAAACGCCCTCCGTTTTGTT	CAACGATCGTGGTGCTGCC	500	0	n.d.	n.d.	In-house
<b>trop 3F/3R</b>	<i>C. tropicalis</i>	AGAGGATTTACCAGCACACAAGA	TCCAGAGATCGGATGATCCAGTAT	500	0	500	0	In-house
CPA 1/2	<i>C. parapsilosis</i>	GCCAGAGATTAACACTCAACCAA	CCTATCCATTAGTTTATACTCCGC	50	1	50	1	Hsu et al 2003
CKRU 1/2	<i>C. krusei</i>	GCATCGATGAAGAAGCCAGC	AAAAGCTAGTTCGCTCGGGCC	50	7	n.d.	n.d.	Hsu et al 2003
CGU 1/2	<i>C. guilliermondii</i>	GCATCGATGAAGAAGCCAGC	GTTTGGTTGTTGTAAGGCCGGG	50	2	n.d.	n.d.	Hsu et al 2003
CGU 3/4	<i>C. guilliermondii</i>	AGTCGGACTAGGCGTTTGCTT	CCCGGCCCTTACAACAACCAAAAC	50	7	n.d.	n.d.	In-house
CGU 5/6	<i>C. guilliermondii</i>	CGGGGAGGTAGTGACAATAC	ATTGCCCCCTTGTTGGTGTGTTG	50	2	n.d.	n.d.	Yamamura et al 2009
<b>CDU 1/2</b>	<i>C. dubliniensis</i>	CTGGCGTCGCCCATTTTATT	GCCACCCCGAAAGAGTAACT	100	0	100	0	In-house
CDU 3/4	<i>C. dubliniensis</i>	GTATTTGCTGTTCCCCCTTTC	GTGTTGTGTCACCTAACGTC	No amplification	–	n.d.	n.d.	Sardi et al 2011

**Notes:** <sup>a</sup>Primer name in boldface indicates the final selection of primer sets. <sup>b</sup>Specificity test was performed against a control panel of 5 ng of genomic DNA from 2 *Aspergillus* sp., 7 common bacteria, human PBMC and other non-target *Candida* species. Numbers shown represent the non-specific amplicons observed. <sup>c</sup>n.d. = not done.





not possible in our study since the regions conserved within *Candida* but distinct from *Aspergillus* are about several hundred base pairs apart on 18S, 5.8S and 28S ribosomal genes. Also, it is known that the melting profiles of an amplicon are less sensitive to sequence variation if its length is over 400 bp,<sup>21</sup> which may be detrimental for differentiating the melting profiles for closely related species. To ensure the shorter amplicon length, we selectively designed the primer sets to amplify ITS-1 and ITS-2 separately as two amplicons so that each one is below 400 bp. Additionally, the reliability of species identification was higher when two independent amplicons were done simultaneously for cross-checking.

It is interesting to note that some of the amplicons produced by our two in-house primer sets had multiple melting peaks for some species, indicating the presence of multiple domains in these particular amplicons. However, they are not off-target amplification as the identities of these amplicons, each as a single band on agarose gel, were confirmed by Sanger sequencing. We also observed that there were some slight variations within species where multiple strains were used. Nonetheless, it did not affect the overall melting profiles for clear species-specific differentiation among the eight *Candida* species. It is possible to further enhance the separation of melting profiles for amplicons targeting ITS-1 or ITS-2 using high-resolution melting analysis. However, we believe that it is sufficient to differentiate eight *Candida* species based on the melting profiles under the resolution provided by SYBR Green dye. Additionally, we have selected eight highly sensitive and species-specific primer sets to cover four major *Candida* species—*C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. dubliniensis*—to further ensure the accuracy of species identification.

## Conclusion

We have successfully demonstrated that the eight medically important *Candida* microbes could be unequivocally differentiated based on their combined melting profiles using the two selected pan-*Candida* primer sets in real-time PCR analysis, with a sensitivity of 10–100 fg DNA. These two primer sets do not amplify excessive *Aspergillus* DNA (1–100 ng) and thereby reduce the potential false positivity due to this common environmental contaminant. The real-time PCR assays developed in this study could potentially be used for the rapid detection and differentiation of *Candida* species in cornea transportation medium or washes before the culture results are available.

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## Author Contributions

Conceived and designed the experiments: GCH, SCL. Performed experiments: JZ, KN and BL. Analyzed the

data: GCH, JZ, KN, ST, SCL. Wrote the first draft: JZ, GCH. Contributed to the writing of the manuscript: GCH, JZ, KN, BL, ST, SCL. Agree with manuscript results and conclusion: GCH, JZ, KN, BL, ST, SCL. Jointly developed the structure and arguments of the paper: GCH, JZ. Made critical revisions and approved final version: SCL. All authors reviewed and approved of the final manuscript.

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